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Purification and properties of citrate synthase from *Acetobacter europaeus*

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Abstract

Citrate synthase (EC 4.1.3.7) was purified from the acidophilic bacterium *Acetobacter europaeus* to electrophoretic homogeneity. The specific activity was 228 units/mg of protein during the exponential ethanol-oxidation growth phase. The enzyme has a molecular mass of 280 kDa and is a hexamer with a subunit size of 46 kDa. The apparent K_m values were 20 μM for oxaloacetate and 51 μM for acetyl-CoA. Unlike citrate synthase from other Gram-negative bacteria, the activity of the enzyme was inhibited by ATP, slightly enhanced by ADP and not effected by NADH. Acetate caused activation of the enzyme. The pH optimum on the citrate synthase activity in vitro was 8.1. The amino-terminal amino acid sequence of the purified enzyme was ENGKSATISLNGKDVALPVL.

Keywords: *Acetobacter europaeus*; Acetic acid; Citrate synthase

1. Introduction

The acetic acid bacteria are well characterized by their ability to oxidize ethanol to acetic acid by membrane-bound alcohol and aldehyde quinoprotein dehydrogenases which are tightly linked to the aerobic respiratory chain [1]. For the metabolism of sugars in the cytoplasm, they possess the pentose phosphate pathway. *Acetobacter* is in contrast to *Gluconobacter* equipped with a complete tricarboxylic acid (TCA) cycle [2]. The TCA and glyoxylate cycle enzyme citrate synthase (EC 4.1.3.7) catalyses the irreversible reaction of acetyl-coenzyme A, oxaloacetate and H_2O to form citrate, free CoA and H^+. Citrate synthase plays a central role in the cell metabolism due to the conservation of energy and the breakdown of organic acids.

The industrially important *Acetobacter europaeus* species is responsible for the production of vinegar with high acidity (7–15% acetic acid) and requires acetic acid for growth [3].

Three genes are reported to be involved in expression of acetic acid resistance in *A. aceti*, with one of these genes (aarA) encoding citrate synthase [4,5].

It was therefore of interest to characterize the citrate synthase from *A. europaeus* and in particular to investigate whether the enzyme from the acidophilic bacterium is similar to that reported from other sources.

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2. Materials and methods

2.1. Bacterial strain

The type strain *A. europaeus* DSM 6160 was obtained from the DSM culture collection (Braunschweig, Germany). *A. europaeus* was cultivated at 30°C in a laboratory fermenter type L 1523 (Bioengineering, Wald, Switzerland) in 10 l of ethanol-acetate medium with an air supply of 500 l/h and at a rate of rotation of 400 rpm. The medium was composed of yeast extract (5 g/l), peptone (3 g/l), 42 mM D-glucose, 495 mM ethanol and 500 mM acetic acid. Antifoam concentrate A (0.5 ml/10 l, Sigma) was used to prevent formation of foam. Cells from 9.5 l culture medium were directly harvested by centrifugation (Sepatech centrifuge 17RS, Heraeus) at $6500 \times g$ at 4°C and a flow rate of 200 ml/min.

2.2. Assays

The activity of citrate synthase was assayed spectrophotometrically by the method of Srere [6]. The reaction mixture contained in a volume of 1 ml 0.1 mM 5.5-dithiobis(2-nitrobenzoic acid), 0.3 mM acetyl-CoA, 0.5 mM oxaloacetate, 190 mM Tris-HCl pH 8.0 and enzyme. One unit of citrate synthase activity is defined as that quantity of enzyme which catalyses the formation of 1 μmol of CoA per min.

Protein was measured by using the Bio-Rad (München, Germany) protein assay kit I under standard and microassay conditions as described by the supplier.

The amounts of D-glucose, ethanol, acetic acid and citric acid were determined enzymatically using test kit combinations from Boehringer Mannheim.

The Michaelis constants (K_m values) were obtained from purified *A. europaeus* citrate synthase by use of the citrate synthase assay conditions. When altering the concentration of any one of the substrates, the concentrations of the other components were maintained at saturation level.

2.3. Preparation of protein extracts

The harvested cells were washed 4–6 times at 4°C in 20 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA, 10 mM $MgCl_2$, 2 mM 1,4-dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (hereafter referred to as buffer A). The cell suspension was disrupted by 10 passages through a French press at 120 MPa. The homogenate was centrifuged at $25\,000 \times g$ for 30 min at 4°C to remove cell debris and to obtain a supernatant of crude enzyme solution.

2.4. Purification of citrate synthase

All purification steps (1–5) were performed at 4°C.

2.4.1. Step 1: Fractionation with $(NH_4)SO_4$

Solid ammonium sulfate was added to the crude enzyme solution. The 55 and 70% ammonium sulfate precipitates were collected by centrifugation for 20 min at $25\,000 \times g$. The precipitate was dissolved in buffer A and dialyzed against the same buffer.

2.4.2. Step 2: Ion exchange chromatography I

The dialyzed enzyme solution was applied to a column (12 cm × 2.6 cm) containing DEAE-Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) which was equilibrated with buffer A. Elution was achieved in four steps with 20, 50, 100 and 150 mM KCl in buffer A at a flow rate of 1 ml/min. The fractions containing citrate synthase and eluting at 20 mM KCl in buffer A were pooled and concentrated by a centriprep 10 concentrator (Amicon, Beverly, USA). The concentrated sample was dialyzed against 20 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, 2 mM DTT and 0.2 mM PMSF (hereafter referred to as buffer B).

2.4.3. Step 3: Gel filtration

The dialyzed sample was applied to a column (48 cm × 1.0 cm) of Sephacryl S-200 HR (Pharmacia Biotech) which was equilibrated with buffer B. Proteins were eluted with 50 ml buffer B at a flow rate of 0.2 ml/min. Selected pooled fractions containing citrate synthase were concentrated and dialyzed against 20 mM Tris-HCl buffer pH 8.0 containing 1 mM EDTA, 10 mM $MgCl_2$, 2 mM DTT and 0.2 mM PMSF (hereafter referred to as buffer C).

2.4.4. Step 4: Ion exchange chromatography II

The product from gel filtration step was loaded

onto a column (5 cm × 5 mm) of Mono Q HR 5/5 matrix (Pharmacia Biotech) by the use of an FPLC system (Pharmacia Biotech). Proteins were eluted by a linear gradient of KCl (0–200 mM) in buffer C with a flow rate of 1 ml/min. The fractions containing citrate synthase were pooled and concentrated by membrane filtration.

2.4.5. Step 5: Hydrophic interaction chromatography

Selected pooled fractions of *A. europaeus* citrate synthase were dialyzed against 20 mM sodium phosphate buffer pH 7.2 containing 30 mM KCl, 0.5 mM $MgCl_2$, 2 mM DTT and 0.2 mM PMSF (hereafter referred to as buffer D). The sample was applied to three columns (bed volume of one column was 1 ml) of Phenyl-Sepharose HiTrap (Pharmacia Biotech) matrix connected in series: High performance, 6 Fast Flow low sub and 6 Fast Flow high sub by the use of an FPLC system (Pharmacia Biotech). The columns were equilibrated with buffer D containing 1 M NaCl. Elution was achieved with a linear gradient of buffer D containing NaCl (1 M–0 M) at a flow rate of 0.8 ml/min.

2.5. Polyacrylamide gel electrophoresis of protein

SDS-PAGE was performed on a 10% polyacrylamide slab gel with Tris-HCl pH 8.3 discontinuous buffer system. Native gel electrophoresis was performed as described by Hames [7]. Protein bands were observed by staining with Coomassie Blue.

2.6. Molecular mass determination

The molecular mass of the purified citrate synthase was determined by gel filtration. A column (48 cm × 1.0 cm) with a Superdex 200 HR 10/30 (Pharmacia Biotech) matrix was connected to a FPLC system (Pharmacia Biotech) and equilibrated with 50 mM potassium phosphate buffer pH 7.2 containing 150 mM NaCl. The standard proteins used for the calibration of the column were blue dextran 2000, thyroglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa). The proteins were eluted with a flow rate of 0.5 ml/min with the buffer used for equilibration. The K_{av} value for each protein was calculated according to the equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the protein, V_0 is the elution volume of blue dextran and V_t is the total bed volume.

2.7. Amino acid analysis

Protein bands were blotted from SDS-PAGE on PVDF membrane (Millipore, Bedford, MA, USA) and directly analysed on an Applied Biosystem 476A (Foster City, CA, USA) Protein Sequencer equipped with a ProBlott cartridge. Sequencing analysis following Edman degradation was run according to the standard protocols provided by the manufacturer.

3. Results

A. europaeus cells from the exponential ethanol-oxidation phase were harvested at an optical density (620 nm) of 0.6, an ethanol concentration of 171 mM, an acetic acid concentration of 883 mM and a glucose concentration of 28 mM. Citric acid was not excreted from *A. europaeus* into the growth medium.

The citrate synthase of *A. europaeus* was purified 600-fold to electrophoretic homogeneity, resulting in an overall increase in specific activity from 0.38 to 230 units/mg protein. The purification scheme of the

Table 1
Purification steps of citrate synthase from *Acetobacter europaeus*

Purification step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
1. Crude extract	113	43	0.4	1.0	100
2. $(NH_4)_2SO_4$	44.4	24	0.5	1.4	55
3. DEAE-Sepharose	1.0	18	19	50	42
4. Sephacryl S-200	0.2	9.6	47	124	22
5. Mono Q	0.1	9.5	95	250	22
6. Phenyl-Sepharose	0.01	2.3	230	600	5

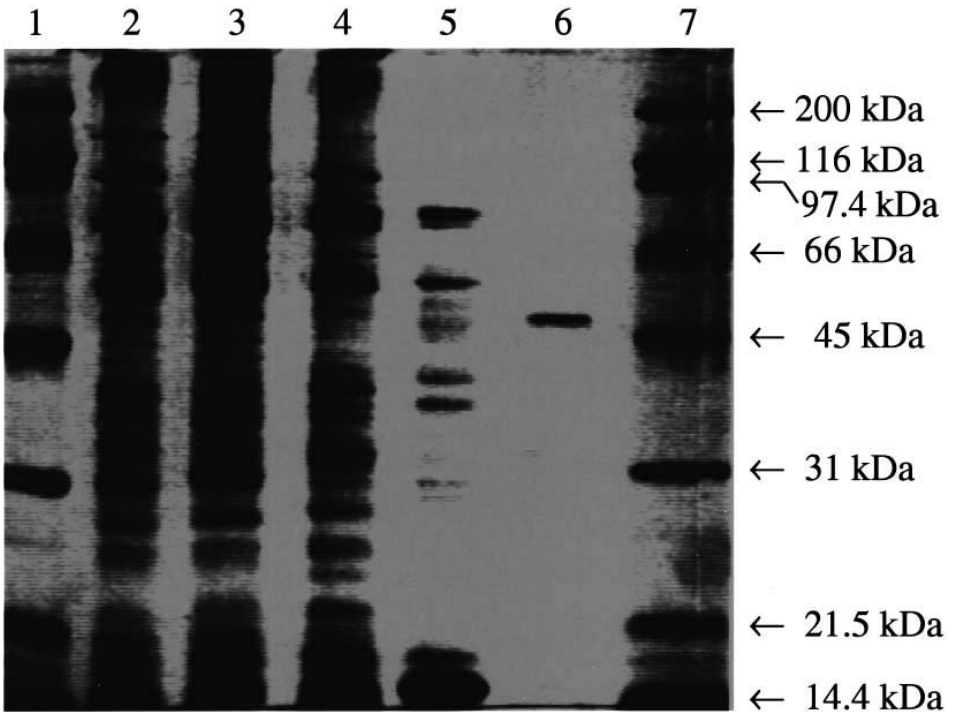

Fig. 1. SDS-PAGE profile of proteins monitoring the purification of the citrate synthase of *Acetobacter europaeus* from the exponential ethanol-oxidation phase. A summary of the purification of the enzyme is given in Table 1. Lane 1, protein standard; lane 2, proteins of crude extract; lane 3, proteins of dissolved $(NH_4)_2SO_4$ precipitate; lane 4, proteins of DEAE-Sepharose eluate; lane 5, proteins of Sephacryl S-200 eluate; lane 6, 1 μg of purified enzyme of Phenyl-Sepharose eluate; lane 7, protein standard (molecular masses: 200 kDa, myosin; 116 kDa, β-galactosidase; 97.4 kDa, phosphorylase *b*; 66 kDa, bovine albumin; 45 kDa, egg albumin; 31 kDa, carbonic anhydrase; 21.5 kDa, trypsin inhibitor; 14.4 kDa, lysozyme).

A. europaeus citrate synthase is shown in Table 1. The SDS-PAGE profile of proteins at different steps of purification is shown in Fig. 1. The purified citrate synthase gave a single protein band and, when compared with the molecular mass standards, a subunit size of 46 kDa was obtained. Upon native gel electrophoresis, the purified enzyme migrated as a single protein. The molecular mass of the native enzyme was determined by gel filtration. The analysis of the corresponding calibration curve revealed a calculated K_{av} value of 0.45 for the *A. europaeus* citrate synthase corresponding to a molecular mass of 280 kDa. These data indicate that the enzyme is a hexamer constituted of six similar subunits.

The substrate velocity curves of the citrate synthase were examined for oxaloacetate and acetyl-CoA. The reaction rates with the purified enzyme were hyperbolic functions of both oxaloacetate and acetyl-CoA. The apparent K_m values of the purified enzyme calculated from the Lineweaver-Burk plots were 20 μM for oxaloacetate and 51 μM for acetyl-CoA. The K_m value of either substrate is independent of the concentration of the other.

Different intermediate metabolites were tested for their effect on the *A. europaeus* citrate synthase activity. ATP at concentrations of 5 and 10 mM produced 15 and 25% inhibition, respectively. The addition of 2.5 mM ADP enhanced the activity of the citrate synthase by 15%. AMP, NAD, NADP, NADH and NADPH (at concentrations of 0.5–5 mM) did not affect the activity of the enzyme. The effect of acetate on enzyme activity was examined. Acetate activated the enzyme significantly, resulting in 27% activation at a concentration of 62 mM.

The N-terminal amino acid sequence of the *A. europaeus* citrate synthase was determined by Edman degradation up to 20 amino acid residues: ENGKSATISLNGKDVALPVL.

The effect of pH in the range 5.0–9.0 on the activity of the *A. europaeus* citrate synthase was examined under the standard assay conditions. The enzyme exhibited optimal activity in vitro at pH 8.1. The purified enzyme was stable at −20°C in 15% glycerol for several weeks.

4. Discussion

The citrate synthase from Eucarya, Archaea and Gram-positive bacteria is an isosterically regulated dimeric enzyme of 100 kDa, whereas the majority of Gram-negative bacteria possess an allosterically regulated hexamer with a molecular mass of the native enzyme of 240–300 kDa [8]. Differences in the polymeric composition of citrate synthase from Gram-negative bacteria have been reported [9–11]. The molecular mass and subunit structure of the *A. europaeus* citrate synthase are consistent with the major citrate synthase forms of Gram-negative bacteria. The K_m value for oxaloacetate of *A. europaeus* citrate synthase (20 μM) is different from that of *A. xylinum* (9 μM) [12]. The K_m value for acetyl-CoA of *A. europaeus* citrate synthase (51 μM) is within the lowest range of the corresponding values from Gram-negative bacteria and is different from that of *A. xylinum* (18 μM) [12]. The kinetic properties of the *A. europaeus* citrate synthase differ from those of Gram-negative bacteria. Citrate synthase of Gram-negative bacteria is feedback inhibited by NADH but not by ATP [13]. The citrate synthase of *A. europaeus* was inhibited by ATP and was not affected by NADH and NADPH. This property is consistent with the findings in *A. xylinum* [12] and *Methylobacillus flagellatum* [9]. Due to the purification to electrophoretic homogeneity of our protein, the specific activity of the *A. europaeus* citrate synthase was 7-times higher than in *A. xylinum*. Unlike other Gram-negative bacteria, the activity of the *A. europaeus* citrate synthase was slightly enhanced by ADP and activated by acetate. From the characterization data of the *A. europaeus* citrate synthase, it is obvious that the enzyme plays a central role in the supply of sufficient ATP to protect the cells from acetic acid and thus correlates indirectly with acetic acid resistance as described by Fukaya et al. [4].

The N-terminal amino acid sequence of the *A. europaeus* citrate synthase is with 7 out of 20 residues identical to the translated amino acid sequence from the citrate synthase gene of *A. aceti* [4]. However, our sequence corresponds to amino acids 8–27 from the protein sequence of *A. aceti* citrate synthase. It is possible that the first seven amino acids were proteolytically removed during the purification procedure of our enzyme.

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